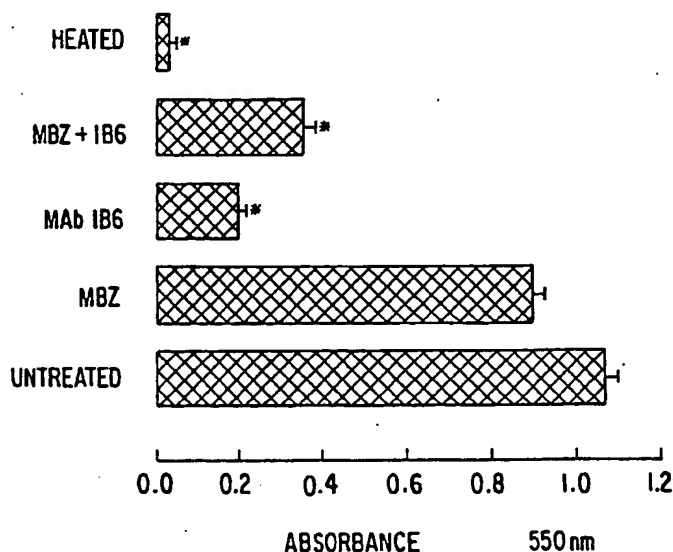




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(54) Title: PEPTIDES AND VACCINES DERIVED FROM NEMATODE TUBULIN**(57) Abstract**

The present invention relates to a monoclonal antibody which substantially binds to β -tubulin of nematode origin and fragments thereof. There is provided a hybridoma cell line producing the monoclonal antibody of the present invention which has been deposited at the ATCC under the accession number HB 11129. The antibody of the present invention can be used as an anti-parasitic agent and a diagnostic agent for parasitic diseases. The present invention also relates to the use of an antigen, which is recognized by the monoclonal antibody, as an immunizing agent and in vaccine compositions.

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PEPTIDES AND VACCINES DERIVED FROM NEMATODE TUBULIN

BACKGROUND OF THE INVENTION1. Field of the invention

The present invention relates to a monoclonal antibody which specifically binds to β -tubulin of nematode origin, which antibody can be used as an anti-parasitic agent and a diagnostic agent for parasitic diseases.

The present invention also relates to the use of immunogenic peptides useful in vaccine compositions for protecting mammals, such as humans and canines, against parasites of the *Brugia* and *Dirofilaria* genuses.

The present invention relates to the use of peptide fragments which provide improved means to protect a mammal against parasites of the *Brugia* and *Dirofilaria* genuses. More specifically, a dog exposed to the peptide fragments of the present invention is protected from heartworm by cytotoxic antibodies induced by the peptide.

2. Brief description of Prior Art

Parasitic diseases such as schistosomiasis (Bilharziasis) malaria and filariasis affect large numbers of people and are frequent causes of gastrointestinal, circulatory and other disorders. Parasitic infections often are chronic or recurrent, and it is not surprising that immunologic types of diseases have been described.

Filariasis consists of a group of diseases occurring in tropical and subtropical countries and caused by *Filarioidea*.

Filariasis involves the lymphatic system, with obstruction leading to chyluria, hydrocoele, and elephantiasis that may involve the scrotum, legs and the arms.

Etiology and Pathogenesis of Filariasis

Wuchereria bancrofti is found only in humans; *Brugia malayi* is often spread to man from animal hosts. The adult filarial worms live in the human lymphatic system. Microfilariae released by gravid females are found in the peripheral blood, usually at night. Infection is spread by many species of mosquitoes. The microfilariae are ingested by the mosquito, undergo development in the insect's thoracic muscles, and, when mature, migrate to its mouthparts. When the infected mosquito bites a new host, the microfilariae penetrate the bite puncture and eventually reach the lymphatics, where they develop to the adult stage.

Pathology

Inflammation and fibrosis occurring in the vicinity of the juvenile and adult worms produce progressive lymphatic obstruction.

Symptoms and signs

The incubation period may be as short as two months. The "prelatent" period, from the time of infection to the appearance of microfilariae in the blood, is at least eight months. Clinical manifestations depend on the severity of the infection; they may include lymphangitis, lymphadenitis, orchitis, funiculitis, epididymitis, lymph varices, and chyluria. Chills, fever, headache, and malaise may also be present. Elephantiasis and other late severe sequelae occur with long-time residence in endemic areas and repeated reinfection. An aberrant form of filariasis (tropical eosinophilia) is characterized by hypereosinophilia, presence of microfilariae in the tissues but not in the blood, and high titers of antifilarial antibodies (tropical eosinophilia). Clinically, the patient may present with lymphadeno-splenomegaly or with cough, bronchospasm, and chest infiltrates.

Diagnosis

Microfilariae may be found in blood or lymph fluid. A number of serologic tests are available, but are not completely reliable. Antigen detection procedures are being investigated.

Tubulin

Microtubules are proteinaceous organelles that are implicated in a variety of cellular functions including mitosis, intracellular transport, the maintenance of cell shape and the formation of cilia, flagella and sensory organelles. The major structural component of microtubules is tubulin, which is composed of α - and β -subunits, the dimer having a molecular weight of 110 kDa. Both α - and β -tubulins are expressed as heterogeneous but closely related families of multiple isoforms, in different organisms, tissues and even within single cells of the same organism. The heterogeneous population of tubulin isoforms may result from both the differential expression of distinct tubulin genes and post-translational modifications. It has been suggested that the diversity in tubulin isoforms may have implications for specific MT functions (Lewis and Cowan, J. of Cell Biol., 1988, 106:2023-2033). The precise nature or role of α - and β -tubulin isoforms have not yet been elucidated, although several groups of researchers have demonstrated that many *in vivo* functions of tubulin are to some extent, isoform specific (Gundersen et al., Cell, 1984, 38:779-789).

Benzimidazoles, anti-mitotic and anti-fungal agents are widely used in the chemotherapy of parasitic diseases. Several chemicals such as colchicine, vinblastine and benzimidazoles have been shown to bind to tubulin. Benzimidazoles exert toxic effects on nematodes by binding to tubulin and inhibiting polymerization of the heterodimer into microtubules. Benzimidazoles induce paralysis and slow growth in the free-living nematode *Caenorhabditis elegans*. These drugs are potent filaricides

for *B. pahangi* and *B. malayi*. However, the precise benzimidazoles binding site has not been determined.

Monoclonal antibodies have made it possible to recognize different domains of tubulin in different species in order to study the structure, distribution and functions of tubulin. Tang and Prichard (Mol. & Biochem. Parasitology, 1989, 32: 145-152) reported the presence of 4 to 5 β -tubulin isoforms in the tubulin-enriched extracts of adult *B. pahangi*. In addition, immunogold studies with *B. malayi* adult and microfilariae using anti-tubulin monoclonal antibodies have revealed the presence of β -tubulin in the somatic muscle blocks beneath the cuticle, intestinal brush border and intra-uterine microfilariae of the adult worms (Helm et al., Parasite Immunology, 1989, 11:479-502).

Several other anti-tubulin monoclonal antibodies raised against parasitic protozoa and nematodes have been isolated but these have been found to cross-react with tubulin from other species. For example, Draber et al (Protoplasma, 1985, 128: 201-207) reported a monoclonal antibody raised against pig brain tubulin which reacted with microtubules from diverse species (mammalian, bird, amphibian, fungi, echinoderm, platyhelminth, slime moulds) but not protozoan tubulin. Similarly, Birkett et al. (FEBS Letters, 1985, 187: 211-218) generated an anti- β -tubulin monoclonal antibody against *Physarum myxamoebae* which reacts with β -tubulin from various fungi, algae, higher plants, avian, insect and several mammalian sources. In addition, Helm et al. (Parasite Immunology, 1989, 11: 479-502) have raised monoclonal antibodies against microfilariae of *Brugia* species. Contrary to the anti-*B. pahangi* β -tubulin monoclonal antibodies of the present invention, their monoclonal antibodies cross-reacted with mammalian tubulin.

All these monoclonal antibodies of the prior art are not specific against tubulin of nematode origin.

It would be highly desirable to have a monoclonal antibody which specifically binds to nematode tubulin and

which could be used as an anti-parasitic agent and as a reliable diagnostic agent for parasitic diseases.

It would be also highly desirable to have a peptide which can be used to immunize mammals against parasites such as *Brugia* and *Dirofilaria*.

The desired peptide could be used in vaccine composition to provide an immune protection against these parasites.

SUMMARY OF THE INVENTION

In accordance with the present invention there is provided a monoclonal antibody which specifically binds to β -tubulin of nematode origin and fragments thereof. The monoclonal antibody of the present invention can be used as an anti-parasitic agent and as a diagnostic agent for parasitic diseases.

In accordance with the present invention, there is also provided a hybridoma cell line which produces the monoclonal antibody of the present invention.

The monoclonal antibody of the present invention recognizes the C-terminal of nematode β -tubulin which corresponds to a peptide of eighteen amino acids.

In accordance with another embodiment of the present invention, there is provided the use of a peptide as an immunizing agent against parasites wherein said peptide has the following amino acid sequence:

Asp	Glu	Glu	Gly	Asp	Leu	Gln	Glu	Gly	Glu	Ser	Glu	Tyr	Ile	Glu	Gln
1				5				10						15	

Glu Glu, SEQ ID NO:1.

The use of the peptide in accordance with the present invention induces by a host the production of cytotoxic antibodies against parasites such as *Brugia* and *Dirofilaria*.

In accordance with the present invention, there is also provided a vaccine for parasite infection comprising a peptide which has the following amino acid sequence:

Asp	Glu	Glu	Gly	Asp	Leu	Gln	Glu	Gly	Glu	Ser	Glu	Tyr	Ile	Glu	Gln
1				5				10						15	

Glu Glu, SEQ ID NO:1.

Finally, in accordance with the present invention, there is provided a method of immunizing mammals against parasites comprising the administration of the vaccine of the present invention. The vaccine of the present invention can be administered in a dosage range of 0.015 μ g to 0.15 mg per kg body weight, preferably in a dosage range of 1.5 μ g to 15 mg per kg body weight.

BRIEF DESCRIPTION OF THE DRAWINGS

Having thus generally described the nature of the invention, reference will not be made to the accompanying drawings, showing by way of illustration a preferred embodiment thereof, and wherein:

Fig. 1 is a Western blot analysis of extracts from parasites, pig brain and 3T3 fibroblasts using anti-*B.pahangi* β tubulin monoclonal antibody in Figure 1A and anti-chick brain tubulin monoclonal antibody in Figure 1B;

Fig. 2 is a Western blot analysis of the total protein extract of adult *B.pahangi* using anti-*B.pahangi* β tubulin monoclonal antibody P3D in Figure 2A or anti-*B.pahangi* β tubulin monoclonal antibody 1B6 in Figure 2B;

Fig. 3 is a Western blot analysis of products from limited proteolysis of anti-*B.pahangi* β tubulin monoclonal antibody P3D in Figure 3A and anti-*B.pahangi* β tubulin monoclonal antibody 1B6 in Figure 3B;

Fig. 4 is a graph of the effects of anti-*B.pahangi* tubulin monoclonal antibody P3D on the viability of adult female *B.phangi* *in vitro*;

Fig. 5 is a graph of the effects of anti-*B.pahangi* tubulin monoclonal antibody 1B6 on the viability of adult female *B.phangi* *in vitro*;

Fig. 6 is a graph of the effects of anti-chick brain tubulin monoclonal antibody 357 on the viability of adult female *B.phangi* *in vitro*.

-6A-

DETAILED DESCRIPTION OF THE INVENTIONI-Monoclonal Antibody

A first embodiment of the present invention relates to the production and characterization of a monoclonal anti-*B. pahangi* tubulin monoclonal antibody.

The monoclonal antibody of the present invention, denoted P3D, specifically reacts to the C-terminal portion of β -tubulin from *B. pahangi* and *Dirofilaria* and

hence is capable of killing these parasites. The hybridoma P3D producing the monoclonal antibody of the present invention has been deposited at the American Type Culture Collection (12301 Parklawn Drive, Rockville, Maryland, USA 20852) under accession number HB 11129 on September 18, 1992. This deposit is available to be public upon the grant of a patent to the assignee, McGill University, disclosing same. The deposit is also available as required by Foreign Patent laws in countries wherein counterpart applications are filed.

In total, fifty-four anti-*B. pahangi* tubulin monoclonal antibodies were obtained after immunization of mice with purified *B. pahangi* tubulin. Because of their remarkable specificity for tubulin, monoclonal antibodies P3D and 1B6 among others, have been selected for more extensive characterization. Western blot analysis of one-dimensional SDS-PAGE showed that the anti-*B. pahangi* monoclonal antibodies of the present invention recognized tubulin from a number of filarial nematodes (*B. pahangi*, *B. malayi* and *D. immitis*) and an intestinal nematode (*H. contortus*). However, the monoclonal antibodies did not cross-react with tubulin from pig brain, 3T3 mouse fibroblast cells or the parasitic protozoan *G. muris*. On the other hand, anti-chick monoclonal antibody 357 reacted with pig brain, 3T3 mouse fibroblast and *G. muris* tubulins as strongly as it did with filarial and other nematode β -tubulins. The anti-*B. pahangi* tubulin monoclonal antibodies of the present invention recognize an epitope that is conserved between filarial and intestinal nematode β -tubulin but not in protozoan and mammalian β -tubulin. Whereas, cross-reactive anti-chick monoclonal antibody 357 recognizes an epitope that is conserved among filarial and intestinal nematodes as well as protozoan and mammalian β -tubulin. The epitope recognized by monoclonal antibody 357 has been localized to a region of β -tubulin between amino acid 339-417 in the proteolytic fragments of pig brain tubulin (Serrano et al., Analytical Biochemistry, 1986, 159: 253-259). The anti-*B. pahangi* tubulin monoclonal antibodies of the

present invention are highly specific to nematode tubulin.

The monoclonal antibodies of the present invention specific for the α - or β -subunit of tubulin allow the subcellular localization and the function of each subunit of tubulin to be studied. Proteins of the size of tubulin are generally built of several structural domains that have distinct functions. In the case of tubulin, such functions include binding of anti-microtubule drugs, GTP or microtubule-associated proteins and the association between monomers, dimers or protofilaments. The nematode-specific anti-tubulin monoclonal antibodies of the present invention may serve to characterize the structure and distribution of *B. pahangi* tubulin molecule, and to define microtubule stability and functional domains.

The following procedures are used in the preparation and the characterization of the monoclonal antibody of the present invention.

Enzyme-linked immunosorbent assay (ELISA)

ELISA was performed in microtiter plates (Falcon) coated with the polylysine-purified tubulin or an 18 amino acid peptide corresponding to the extreme C-terminal residues 430-448 of *B. pahangi* tubulin (Guénette et al., Mol. and Biochem. Parasitology, 1991, 44: 153-164) at a concentration of 10 μ g/ml in phosphate buffer saline (PBS). Plates are incubated with 200 μ l of 1% bovine serum albumin (BSA) in PBS. Horseradish peroxidase-labeled anti-mouse IgG or IgM (Bio-Can, Mississauga, Ontario) at dilutions of 1:5000 and 1:20,000, respectively, is added to each well and incubated for 1 hour at 37°C. The substrate is 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (Sigma). The plates are read on a Titertek multiskan™ plate (Flow Laboratories, Irvine, Ayrshire, UK) at 414 nm. Normal mouse serum or culture medium used to grow hybridoma cells (Iscoves modified Dulbecco's medium (IMDM) with 20% FCS, 10% NCTC 135 and HT) is used as a negative control.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Samples are run in a Mini Protean II™ dual slab cell (Bio-Rad, Richmond, CA) using 4% polyacrylamide as stacking and 12% polyacrylamide as separating gels.

Isoelectric focusing and two-dimensional electrophoresis (IEF-2D SDS-PAGE)

IEF gels are prepared and run in tube gels (1.5 x 8cms) containing 9.5M urea (LKB) and 2% (w/v) ampholines (LKB) (1.6% pH 4-6 and 0.4% pH 3.5-10). IEF is conducted at 400 V for a period of 16 hours and then at 800 V for 3 h. Electrophoresis is performed in 4% polyacrylamide stacking and 12% polyacrylamide separating gels, running at 50 V for 30 min and at 150 V for 60 min, in the Mini Protean II™ slab cell. After 2-dimensional (2D) SDS-PAGE, gels are either stained with silver stain (Bio-Rad), or the proteins are transferred onto nitrocellulose (NC) sheets for Western blot analysis.

Western blotting

After 1 and 2D SDS-PAGE, tubulin subunits, individual tubulin isoforms and peptides are electrophoretically transferred onto nitrocellulose sheets for 2 hours at 4°C. The nitrocellulose sheets are cut into several strips containing an identical pattern of separated proteins. To visualize protein bands, two nitrocellulose strips are stained with amido black. The remaining strips are washed in PBS and incubated for 2 hours at room temperature in 10% newborn calf serum (Gibco) in Tris-buffer saline (140 mM NaCl₂, 50 mM Tris-HCl, pH 7.4, with 0.1% (v/v) Tween 20™ (TBS-T)) to saturate the unoccupied protein binding sites of the nitrocellulose. After washing, the strips are incubated overnight at 4°C with anti-tubulin monoclonal antibodies (MAbs) or IMDM (negative control). The nitrocellulose strips are then washed 6 x 5 min with TBS-T, immersed in peroxidase-conjugated goat anti-mouse IgM or IgG (Bio-

Can) diluted at 1:500 with high salt buffer (1 M NaCl₂, 10 mM Tris-HCl, pH 7.4; 0.5% (v/v) Tween 20™ (HSB-T) with 10% NBCS)), and incubated for 2 hours at room temperature. After washing the nitrocellulose strips with TBS-T for 30 min, the bound peroxidase is detected with the substrate 4-chloro-1-naphthol (Sigma) at 3 mg/ml in methanol/PBS, 1:5 (vol/vol), containing 0.075% of 30% hydrogen peroxide.

1- Preparation of antigens

Gerbils (*Meriones unguiculatus*), 9-10 months old and previously infected intraperitoneally with 400 *B. pahangi* infective larvae, are obtained from Dr. J. McCall (University of Georgia, USA). The adult *B. pahangi* (0.7 g) are harvested from the peritoneal cavities of gerbils in warm physiological saline (0.85% NaCl), washed with 0.025M buffer containing 1 mM ethyleneglycol-bis-(β -aminoethyl ether)N,N,N',N'-tetraacetic acid (EGTA), 0.5mM MgSO₄ and 1 mM guanosine-5'-triphosphate (GTP), and are homogenized in 7 ml of 2[N-morpholino]-ethanesulfonic acid (MES) buffer. The homogenate is centrifuged at 100,000 g for 1 hour at 4°C. The supernatant is retained and the pellet discarded. The same procedure is used to prepare tubulin from other filarial (*B. malayi* and *D. immitis*) and non-filarial nematodes (*A. suum*, benzimidazole-susceptible and resistant strains of *H. contortus*).

Tubulin from pig brain is prepared by 2 cycles of polymerization-depolymerization.

Giardia Muris antigen is prepared as a sonicate.

A peptide corresponding to amino acid residues 430-448 of *B. pahangi* β -tubulin, synthesized using an Applied Bisystems Peptide Synthesizer™, HPLC purified, sequenced and coupled to the carrier protein, keyhole Limpet Hemocyanin (KLH) (The Alberta Peptide Institute), is also used as an antigen in enzyme-linked immunosorbent assay (ELISA).

2- Purification of parasite tubulin

B. pahangi, *B. malayi*, *D. immitis*, *A. suum* and *H. contortus* tubulins are partially purified using polylysine affinity chromatography (Lacey & Prichard, Mol. & Biochem. Parasitology, 1986, 19: 171-181). The elution profile consisted of three distinct peaks. The first protein peak is eluted with MES buffer, the second with 1% aqueous $(\text{NH}_4)_2\text{SO}_4$. Fractions for each peak are pooled and concentrated separately in centriflo™ (Amicon) at 400 g.

Polylysine-purified proteins are separated on SDS-PAGE, protein bands of the molecular weight corresponding to tubulin are excised, and the protein is electro-eluted (Electroeluter™, Bio-Rad) (Blöse et al., J. of Cell Biol., 1984, 98: 847-858). The eluted protein is precipitated three times with 80% acetone at -20°C for 5 hours and then dissolved in 0.125 M Tris-HCl (pH 6.8), 0.1% SDS and 1 mM EDTA, dialysed overnight against this buffer at 4°C and stored at -70°C until used.

Crude supernatant of adult *B. pahangi* is chromatographed on a polylysine agarose column. The protein content of each fraction is determined. The elution profile consisted of 3 distinct protein peaks. The protein concentrations in the first and second peaks are very high compared with that in the third peak, but in contrast to this last peak the first two peaks contained little if any tubulin. This is consistent with the previous report by Tang & Prichard (Mol. & Biochem. Parasitology, 1989, 32: 145-152). Third peak proteins are concentrated and then subjected to SDS-PAGE, respectively. The tubulin band is cut out of the SDS-gels and subjected to electro-elution for further purification.

3- Immunization and preparation of monoclonal antibodies

Six week old female BALB/c mice (Charles River Canada Inc., St. Constant, Québec) are injected subcutaneously at three week intervals with purified eluted *B.*

pahangi tubulin (100 µg/injection) using equal volumes of complete Freund's adjuvant for the first injection and incomplete adjuvant for the second injection. The third immunization of 100 µg of tubulin in PBS is administered intraperitoneally (i.p.). At this stage, mice are bled and serum is tested for anti-tubulin antibodies by ELISA and Western blotting. The spleen cells from the mouse giving the highest titer are fused with the myeloma cell line, P3X63.Ag8 (American Type Culture Collection (ATCC), accession number CRL1580, Rockville, MD), as described by Hurrell ("Monoclonal hybridoma antibodies: Techniques and applications", 1982, CRC Press, Boca Raton, Florida, p. 22). Positive cultures as determined by ELISA and Western blotting, are cloned twice by limiting dilution.

Two different isotypes of anti-*B. pahangi* monoclonal antibodies were obtained. Seven out of fifty-four monoclonal antibodies were polyreactive IgM, recognizing tubulin as well as other high and low molecular weight proteins, whereas the remaining monoclonal antibodies represented two populations of the IgG isotype. Of these, four out of fifty-four reacted with tubulin and other low molecular weight proteins; however, forty-three monoclonal antibodies were specific for tubulin. Monoclonal antibodies P3D and 1B6 specific to nematode tubulin, were chosen for further characterization. These monoclonal antibodies are of IgG isotype.

4- Monoclonal antibodies (MAbs)

Three monoclonal antibodies, all specific for tubulin, are investigated. Anti-chick brain monoclonal antibody 357, which cross-reacts with β -tubulins from a spectrum of eukaryotic cell types, was purchased from the Radiochemical Centre (Amersham, England) and monoclonal antibodies P3D and 1B6 are raised against the tubulin of adult *B. pahangi*. All anti-tubulin monoclonal antibodies are of IgG isotype.

5- Specificity of monoclonal antibodies (MAbs) P3D, 1B6 and 357

The specificity of these monoclonal antibodies is investigated by determining their reactivity to proteins from a variety of filarial and non-filarial nematodes, protozoa and mammalian cells using ELISA and Western blot.

In ELISA, the anti-*B. pahangi* monoclonal antibodies P3D and 1B6 do not react with *G. muris* tubulin, which is recognized by anti-chick brain tubulin monoclonal antibody 357. Crude and partially purified extracts of adults and microfilariae of *B. pahangi*, adult *B. malayi* and *D. immitis*, eggs of *H. contortus*, adult *A. suum*, pig brain and 3T3 mouse fibroblast cell tubulins are separated on SDS-PAGE and electrophoretically transferred onto nitrocellulose sheets. The blots are treated with: (1) amido black; (2) monoclonal antibody 1B6; (3) monoclonal antibody P3D; and monoclonal antibody 357. Analysis of amido black stained blots revealed that crude extracts of adults and microfilariae of *B. pahangi*, adult *B. malayi* and *D. immitis*, eggs of susceptible and resistant strains of *H. contortus*, adult *A. suum*, pig brain and 3T3 mouse fibroblast cell contained many bands in the tubulin region. Tubulin from the various nematodes and mammalian extracts are separated into two bands designated α and β . Anti-*B. pahangi* monoclonal antibody P3D recognized specifically β -tubulin from adult and microfilariae of the filarial worms *B. pahangi*, *B. malayi* and *D. immitis* (Fig. 1A, lane 1-4). It also reacted with equal intensity to tubulin from the intestinal nematode *H. contortus* (BZ-susceptible and benzimidazole-resistant strains) (Fig. 1A, lane 5-6). Tubulin from *A. suum* do not show very strong reactivity with this monoclonal antibody (Fig. 1A, lane 7), no reactivity to 3T3 mouse fibroblast cells or pig brain tubulins is detected (Fig. 1A, lane 8-9). Similar results are obtained using monoclonal antibody 1B6 (not shown). Whereas, cross-reactive anti-chick β -tubulin monoclonal antibody 357 recognized β -tubulin from all nematodes and mammalian cells (Fig. 1B, lane 1 to 9).

6- Identification of tubulin isoforms

Anti-*B. pahangi* β -tubulin monoclonal antibodies P3D and 1B6, and anti-chick β -tubulin monoclonal antibody 357, are used to characterize β -tubulin isoforms in *B. pahangi* tubulin. Monoclonal antibodies P3D (Fig. 2A) and 357 recognized the same isoform pattern, reacting with two β -tubulin isoforms in the crude as well as partially purified extracts of *B. pahangi* (not shown). Whereas, monoclonal antibody 1B6 specifically recognized only one β -tubulin isoform in the extract of *B. pahangi* (Fig. 2B). The β -tubulin isoforms are in the pH range of 5.1-5.3.

Monoclonal antibody 357 probed blots are re-probed with monoclonal antibodies P3D and 1B6 respectively, to demonstrate that the same spots are recognized by this monoclonal antibody. Furthermore, to show the full complement of β -tubulin isoforms, P3D and 1B6 probed blots are re-probed with monoclonal antibody 357. The results indicated that all these monoclonal antibodies recognized the same isoforms in tubulin-enriched extracts of adult *B. pahangi*. However, monoclonal antibody 1B6 is specific to one isoform.

7- Limited Proteolysis of tubulin

Limited proteolysis of tubulin in gel slices is performed. Gel pieces corresponding to the tubulin are cut out of the polyacrylamide gels and placed directly into the sample well of a second 15% SDS-polyacrylamide gel. Gel pieces are overlaid with one of the following proteases: α -chymotrypsin from bovine pancreas (Sigma) or *S. aureus* V8 protease (Boehringer Mannheim). The SDS-PAGE is performed at 50 V until bromophenol blue dye reached the bottom of the stacking gel and then increased to 150 V for the remainder of the electrophoresis. After SDS-PAGE, the digested peptides are either stained with silver stain or transferred onto nitrocellulose sheets, in the same way as described for the Western blot analysis, and reacted either with anti-*B. pahangi* tubulin

monoclonal antibodies or anti-chick tubulin monoclonal antibody 357.

8- Interaction of anti-tubulin monoclonal antibodies with tubulin proteolytic fragments

Three identical gels are run and the peptide fragments transferred onto nitrocellulose, Three of which are immunostained with anti-*B. pahangi* tubulin monoclonal antibodies P3D and 1B6 (Fig. 3) and anti-chick brain β -tubulin monoclonal antibody 357 (not shown).

Western blots of peptides digested with chymotrypsin showed that monoclonal antibody P3D reacted with a 21 kDa chymotrypsin fragment (Fig. 3A, lane 2) and a 21 kDa V8 protease β -tubulin fragment (Fig. 3A, lane 3). In contrast, monoclonal antibody 1B6 reacted with the two chymotrypsin-digested fragments of 42 and 34 kDa (Fig. 3B, lane 2). It reacted strongly with the 42 kDa and weakly with the 34 kDa fragment. However, the same protease (Fig. 3B, lane 3). These results of the limited proteolysis analysis indicate that the antigenic site recognized by monoclonal antibody P3D differs from that recognized by monoclonal antibody 1B6.

Although monoclonal antibody 357 reacts strongly to intact β -tubulin from *B. pahangi*, no interaction was seen with β -tubulin fragments digested with chymotrypsin or V8 protease (not shown). Protease digestion appears to destroy the reactivity of *B. pahangi* tubulin towards monoclonal antibody 357.

II-Peptide used as an immunizing agent against parasite

The monoclonal antibody P3D of the present invention recognizes the C-terminal of nematode β -tubulin which corresponds to a peptide of eighteen amino acids.

A second embodiment of the present invention relates to the use of a peptide, recognized by the antibody of the present invention, which consists of the following eighteen amino acid sequence:

Asp Glu Glu Gly Asp Leu Gln Glu Gly Glu Ser Glu Tyr Ile Glu Gln
 1 5 10 15

Glu Glu, SEQ ID NO:1, which is located at the C-terminal of nematode β -tubulin. Furthermore, the present invention relates to a vaccine which comprises a peptide that consists of the following amino acid sequence:

Asp Glu Glu Gly Asp Leu Gln Glu Gly Glu Ser Glu Tyr Ile Glu Gln
 1 5 10 15

Glu Glu, SEQ ID NO:1.

The present invention provides a peptide having the amino acid sequence derived from the eighteen amino acids at the C-terminal of β -tubulin from *B. pahangi* and *Dirofilaria*. The peptide can be made using a peptide sequence or using recombinant DNA technology.

A vaccine comprising the peptide of the present invention, a fragment thereof or a larger peptide which comprises the amino acid sequence of the peptide of the present invention is effective in conferring protection against parasite infection. Such vaccines can be prepared by one having ordinary skill in the art.

It has been discovered that monoclonal antibodies which specifically react to the C-terminal portion of β -tubulin from *B. pahangi* or *Dirofilaria* are capable of killing these parasites..

Accordingly, using a vaccine that comprises peptide with the epitope of the C-terminal of *B. pahangi* or *Dirofilaria* β -tubulin will elicit cytotoxic antibodies in vaccinated mammals that can kill these parasites and therefore protect the mammal against the parasite.

The present invention relates to vaccines which comprise a peptide which consist of the eighteen amino acid residues from the C terminus of *B. pahangi* or *Dirofilaria* β -tubulin or fragment thereof and to vaccines which comprise a peptide that have portions which are the eighteen amino acid sequence.

The amino acids at the carboxy terminus of *Brugia* and *Dirofilaria* β -tubulin are:

DEEGDLQEGESEYIEQEE or

Asp	Glu	Glu	Gly	Asp	Leu	Gln	Glu	Gly	Glu	Ser	Glu	Tyr	Ile	Glu	Gln
1				5				10						15	

Glu Glu, SEQ ID NO:1, or

aspartate-glutamate-glutamate-glycine-aspartate-leucine-glutamine-glutamate-glycine-glutamate-serine-glutamate-tyrosine-isoleucine-glutamate-glutamine-glutamate-glutamate.

Production of the peptide of the present invention, fragment thereof or larger peptides which include this sequence can be accomplished using standard peptide synthesis or recombinant DNA techniques both well known to those having ordinary skill in the art. Peptide synthesis is the preferred method of making polypeptides which comprise about 50 amino acids or less. For larger molecules, production in host cells using recombinant DNA technology is preferred.

Smaller peptides according to the present invention can be synthesized, for example, by solid-phase methodology utilizing an Applied Biosystems 430A peptide synthesizer (Applied Biosystems, Foster City, California) as described in detail below.

For larger molecules, production in host cells using recombinant DNA is preferred. There are several different methods available to one having ordinary skill in the art who wishes to use recombinant DNA technology to produce proteins. Typically, genes encoding desired polypeptides are inserted in expression vectors which are then used to transform or transfect suitable host cells. The inserted gene is then expressed in the host cell and the desired polypeptide is produced.

Methods and materials for preparing genes and recombinant vectors, transforming or transfecting host cells using the same, replicating the vectors in host cells and expressing biologically active foreign peptides and proteins are described in Principles of Gene Manipulation, by Old and Primrose, 2nd edition, 1981 and Sambrook et al., Molecular Cloning, 2nd Edition, Cold

Spring Harbor Laboratory Press, NY (1989), the disclosure of both is incorporated herein by reference.

European Patent application 322,237 published on June 28, 1989, U.S. Patent 4,735,801 (Stocker, April 5, 1988) and U.S. Patent 4,837,151 (Stocker, June 6, 1989) describe attenuated microorganisms useful in vaccine and which microorganisms have non-reverting mutation in two discrete genes in their aromatic biosynthetic pathway. These microorganisms can usefully form the basis of an oral live vaccine and can be genetically engineered so as to express antigens from other pathogens. These references are all incorporated herein by reference.

Example I

In vitro assay of *B. pahangi* inhibition

Measurement of the *in vitro* activity of anti-*B. pahangi* tubulin monoclonal antibodies P3D, 1B6 and anti-chick brain tubulin monoclonal antibody 357 against female *B. pahangi* or anti-chick tubulin monoclonal antibodies can independently cause any damage to the intact adult worms. Mebendazole (MBZ) is used to determine whether the presence of MBZ drug alone or in synergy with monoclonal antibodies has any differential effect.

Inhibitors

Anti-*B. pahangi* β -tubulin monoclonal antibodies P3D, 1B6 (in culture medium)), anti-chick β -tubulin monoclonal antibody 357 (in ascites fluid) and mebendazole (MBZ) (in DMSO), a benzimidazole anthelmintic drug, are used as inhibitors in the *in vitro* assays. Anti-*B. pahangi* anti-chick brain monoclonal antibody 357 is in ascites fluid and is diluted to 1:1000 concentration with culture medium IMDM/FCS.

Culture *in vitro*

Parasitic nematodes are isolated from their mammalian host. *B. pahangi* are isolated from peritoneal cavities of gerbils, as described earlier in a sterile hood of Iscove's Modified Dulbecco's Medium/NCTC-135

supplemented with 20% fetal calf serum (IMDM/FCS). Following isolation, *B. pahangi* are washed five times with sterile IMDM/FCS medium, for surface sterilization. Three wells in 24-well plates (Nunc) are set up for each test monoclonal antibody, drug and for the control cultures. To each well was added 2 ml of the appropriate test medium containing pure monoclonal antibody P3D, 1B6, 357 alone or monoclonal antibody and MBZ and two adult worms. The plates are incubated at 37°C in a humidified incubator in the presence of 95% air and 5% CO₂. Worm activity is observed every two hours, and motility is assessed subjectively by observation with a naked eye. Experiment is terminated after 48 hours. During the 48 hour incubation the culture medium is not changed. Control medium contained an identical volume of the IMDM/FCS without monoclonal antibodies or drug.

Optimization of MTT reduction assay

Female live *B. pahangi* worm is placed in 0.5 ml of IMDM containing 0.5 mg/ml [3-(4,5-dimethyl(thiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (Sigma) (MTT) and incubated at 37°C for various time intervals ranging from 0-90 min (MTT-reduction). Female worms that had previously been heat-killed are also incubated with MTT for selected time intervals over this range. For each time point three replicate worms are used. At the end of the MTT incubations worms are removed and carefully transferred to a separate well of a microtiter plate containing 200µl of DMSO and allowed to stand at room temperature for 1 hour (formazan solubilization), with occasional gentle agitation to evenly disperse the color. The absorbance of the resulting formazan solution is then determined at 550 nm, using an ELISA reader and compared with a DMSO blank.

Quantification of *B. pahangi* viability

A three-step colorimetric assay based on MTT is used to assess viability of parasitic nematodes. MTT is dissolved in PBS at a concentration of 5.0 mg/ml and sub-

sequently diluted to 0.5 mg/ml with PBS. Worms are incubated for 30 min at 37°C (MTT reduction). After incubation, worms are transferred to 96 well plates containing 200µl of DMSO. The plates are allowed to stand for 1 hour at room temperature (formazan solubilization). The absorbance is determined at 550 nm in the presence and absence of worm and compared with a DMSO blank. Worms are killed for control purposes by heating in PBS at 100°C for 10 min.

Previous studies have demonstrated the utility of MTT-formazan colorimetry in proliferation and cytotoxicity assays in anti-cancer chemotherapy. Subsequently it has been demonstrated that the application of this assay was successful to determine filarial viability and for *in vitro* anti-filarial drug screening. MTT is pale-yellow in solution but when incubated with living cells is reduced by active mitochondria to yield a dark blue crystalline deposit (formazan) within cells, which once solubilized can be quantified colorimetrically.

In accordance with the present invention, MTT assays are performed to determine the effects of anti-tubulin monoclonal antibodies on the viability of parasitic nematodes. Viable control female *B. pahangi* showed rates of formazan formation that are maximal and linear during the first 30 min of the incubation with MTT. By one hour rate of formazan formation had begun to decline and plateaued between 60-90 min. Heat-killed worms shows only background levels of formazan formation.

Worms treated with anti-*B. pahangi* monoclonal antibody P3D alone and in synergy with MBZ show a detectable decrease in motility 12 hours post-treatment. The other anti-*B. pahangi* monoclonal antibody 1B6 alone and in synergy with MBZ, also exhibit an apparent decline in the motility of worms, however, no mortalities are observed using these monoclonal antibodies during the experiment. No noticeable reduction is observed in the motility of the worms treated with MBZ alone or anti-chick brain monoclonal antibody 357 alone or in synergy

with MBZ or the control worms, during the period the worms are in culture. From these observations, it is suggested that the reduction in the worm motility is caused mainly by the anti-*B. pahangi* monoclonal antibody alone, since MBZ alone do not have any effect on the motility of the worms.

Analysis of MTT assays demonstrates that monoclonal antibody P3D treated *B. pahangi* shows significant decline in their ability to reduce MTT to formazan (Fig. 4). This monoclonal antibody alone caused a highly significant 80% reduction in worm viability, compared with untreated live worms, 48 hours post-treatment. MBZ in synergy with monoclonal antibody P3D caused significant 70% reduction in the viability of worms. The high reduction in the viability of worms seems to be due to the presence of monoclonal antibody P3D and not MBZ. As MBZ alone induced a minimal decrease (10%) in the viability of worms. Exposure to monoclonal antibody 1B6 resulted in 40% decrease in the ability of worms to reduce MTT (Fig. 5). Monoclonal antibodies P3D and 1B6 had the same respective effects on the viability of males as for females. Anti-chick β -tubulin monoclonal antibody 357 did not show any significant effect on the viability of worms (Fig. 6). The properties of anti-*B. pahangi* and anti-chick brain monoclonal antibodies appear qualitatively similar. Differences in their inhibitory effects on the motility and viability of *B. pahangi* may depend on their different binding affinities.

Control untreated live female worms show a linear rate of formazan production and gave an absorbance reading of 1.1 at 550 nm. In contrast, heat killed worms show no ability to reduce MTT (Fig. 4 to 6). After DMSO solubilization for 1 hour the absorbance of the resulting formazan solution is determined in the presence or absence of the female worms. This is done to determine if the presence of worm had any effect on the absorbance values. In the presence of worm, there is a slight increase in the absorbance values obtained. Inhibition of MTT reduction does not always occur uniformly along

the entire length of treated worms and areas retaining viability are observed. Thus by close observation of the worms during MTT reduction it is sometimes possible to determine sites of selective damage.

Conclusion

The results of Example I demonstrate an apparent decline in the motility, when the worms are cultured with the anti-tubulin monoclonal antibodies P3D and 1B6 of the present invention. However, no noticeable reduction in the motility is observed, when the worms are treated with anti-chick monoclonal antibody 357, MBZ or IMDM/FCS culture medium without antibodies. The viability of the worms was assessed by MTT assay. The anti-*B. pahangi*, monoclonal antibodies P3D and 1B6 of the present invention, significantly reduced the viability of parasitic nematodes. No reduction in viability was observed when adult *B. pahangi* were exposed to anti-chick monoclonal antibody 357 and/or MBZ.

Example II

Anti-parasitic Antibody composition

An antibody composition to be administered to a gerbil as an anti-parasitic agent in dosage varying from 1mg/0.5ml to 10mg/0.5ml in a pharmaceutical carrier suitable for intraperitoneally administration.

The carrier for such administration is an IMDM culture media.

Example III

Production of the eighteen Amino Acid peptide

The peptide consists of the amino acid sequence:

Asp	Glu	Glu	Gly	Asp	Leu	Gln	Glu	Gly	Glu	Ser	Glu	Tyr	Ile	Glu	Gln
1				5				10						15	

Glu Glu, SEQ ID NO:1.

To prepare the peptide for use in a vaccine, the peptide is synthesized by solid phase methodology on an Applied

Biosystems Inc. (ABI) 430A peptide synthesizer using ABI's Small Scale Rapid Cycles (SSRC) on a 0.1 mmole scale or other similar synthesizer. SSRC utilizes abbreviated single couple cycles with standard Boc chemistry. The t-Boc-L-amino acids used (1 mmole) are supplied by ABI with standard side-chain protecting groups. The completed peptide is removed from the supporting PAM (phenylacetamidomethyl) resin, concurrently with the side-chain protecting groups, by a standard HF procedure using appropriate cation scavengers (10% v/v anisole, p-cresol plus p-thiocresol, 1,4-butanedithiol plus anisole or DMS plus anisole) depending on the amino acid sequence of the peptide.

The crude peptide, after HF cleavage, is purified by preparative reverse phase chromatography on a Phenomenex C-18 Column (250 x 22.5 mm) using water acetonitrile gradients, each phase containing 0.1% TFA. The pure fractions (as determined by analytical HPLC) are pooled, acetonitrile evaporated and the aqueous solution lyophilized. The peptide is analyzed by fast atom bombardment mass spectroscopy and resulting $(M+H)^+$ is compared with the anticipated $(M+H)^+$.

Example IV

Vaccine Comprising Eighteen Amino Acid Peptide

The peptide can be prepared in vaccine dose form by well-known procedures. The vaccine can be administered sublingually, intramuscularly, subcutaneously or intranasally. For parenteral administration, such as intramuscular injection, the immunogen may be combined with a suitable carrier, for example, it may be administered in water, saline or buffered vehicles with or without various adjuvants or immunomodulating agents such as aluminum hydroxide, aluminum phosphate, aluminum potassium sulfate (alum), beryllium sulfate, silica, kaolin, carbon, water-in-oil emulsions, oil-in-water emulsions, muramyl dipeptide, bacterial endotoxin, lipid X, *Corynebacterium parvum* (*Propionobacterium acnes*), *Bordetella pertussis*, polyribonucleotides, sodium

alginate, lanolin, lysolecithin, vitamin A, saponin, liposomes, levamisole, DEAE-dextran, blocked copolymers or other synthetic adjuvants. Such adjuvants are available commercially from various sources, for example, Merck Adjuvant 65 (Merck and Co., Inc., Rahway, NJ).

The proportion of immunogen and adjuvant can be varied over a broad range so long as both are present in effective amounts. For example, aluminum hydroxide can be present in an amount of about 0.5% of the vaccine mixture (Al_2O_3 basis). On a per dose basis, the concentration of the immunogen can range from about 0.015 μg to about 1.5 mg per kilogram per patient body weight for an animal or human patient. A preferable dosage range in humans is about 0.1 - 1 ml, preferably about 0.1 ml. Accordingly, a dose for intramuscular injection, for example, would comprise 0.1 ml containing immunogen in admixture with 0.5% aluminum hydroxide.

The vaccine of the present invention may also be combined with other vaccines for other diseases to produce multivalent vaccines. It may also be combined with other medicaments such as antibiotics.

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modification and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth, and as follows from the scope of the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: McGill University
845 Sherbrooke Street West
Montreal, Quebec, CANADA H3A 1B1
- (ii) TITLE OF INVENTION: Peptides and Vaccines Derived From
Nematode Tubulin
- (iii) NUMBER OF SEQUENCES: 1
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: MERCHANT & GOULD
 - (B) STREET: 3100 Norwest Center
 - (C) CITY: Minneapolis
 - (D) STATE: MN
 - (E) COUNTRY: USA
 - (F) ZIP: 55402
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER:
 - (B) FILING DATE:
 - (C) CLASSIFICATION
- (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: US 07/967,829
 - (B) FILING DATE: 28-OCT-1992
 - (C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: Woessner, Warren D.
 - (B) REGISTRATION NUMBER: 30,440
 - (C) REFERENCE/DOCKET NUMBER: 10022.3-W001
- (ix) TELECOMMUNICATION INFORMATION:
 - (A) TELEPHONE: 612-332-5300
 - (B) TELEFAX: 612-332-9081

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 18 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Asp Glu Glu Gly Asp Leu Gln Glu Gly Glu Ser Glu Tyr Ile Glu Gln
1 5 10 15

Glu Glu

WE CLAIM:

1. A monoclonal antibody which substantially binds to β -tubulin of nematode origin and fragments thereof.
2. A hybridoma cell line producing the monoclonal antibody of claim 1 and deposited at the ATCC under accession number HB 11129.
3. The use of the antibody of claim 1 as an anti-parasitic agent.
4. The use of the antibody of claim 1 as a diagnostic agent for parasitic diseases.
5. The use of claim 3 directed against filariasis-causing parasites.
6. The use of claim 4, wherein the parasitic disease is filariasis.
7. The use of a peptide as an immunizing agent against parasites, wherein said peptide has the amino acid sequence:
Asp Glu Glu Gly Asp Leu Gln Glu Gly Glu Ser Glu Tyr Ile Glu Gln
1 5 10 15
Glu Glu, SEQ ID NO:1, or a fragment thereof.
8. The use of a peptide as an immunizing agent against parasites, wherein said peptide has at least the amino acid sequence:
Asp Glu Glu Gly Asp Leu Gln Glu Gly Glu Ser Glu Tyr Ile Glu Gln
1 5 10 15
Glu Glu, SEQ ID NO:1.
9. The use of a peptide to induce production by a host of cytotoxic antibodies against parasites, which peptide has the amino acid sequence:

Asp Glu Glu Gly Asp Leu Gln Glu Gly Glu Ser Glu Tyr Ile Glu Gln
1 5 10 15

Glu Glu, SEQ ID NO:1, or a fragment thereof.

10. The use of a peptide to induce production by a host of cytotoxic antibodies against parasites, which peptide has at least the amino acid sequence:

Asp Glu Glu Gly Asp Leu Gln Glu Gly Glu Ser Glu Tyr Ile Glu Gln
1 5 10 15

Glu Glu, SEQ ID NO:1.

11. A vaccine for parasite infection comprising a peptide which has the amino acid sequence:

Asp Glu Glu Gly Asp Leu Gln Glu Gly Glu Ser Glu Tyr Ile Glu Gln
1 5 10 15

Glu Glu, SEQ ID NO:1, or a fragment thereof in association with a pharmaceutical carrier.

12. A vaccine for parasite infection comprising a peptide which has at least the amino acid sequence:

Asp Glu Glu Gly Asp Leu Gln Glu Gly Glu Ser Glu Tyr Ile Glu Gln
1 5 10 15

Glu Glu, SEQ ID NO:1.

13. A method of immunizing mammals against parasites comprising the administration of a vaccine according to claim 11 or 12.

14. The method of immunizing of claim 13, wherein the parasite is a nematode.

15. The method of immunizing of claim 14, wherein the nematode is a heartworm and the mammal is a canine.

16. The method according to claim 13, 14 or 15, wherein said vaccine is administered in a dosage range of 0.015 μ g to 0.15 mg per kg body weight.

17. A method according to claim 16, wherein said vaccine is administered in a dosage range of 1.5 μ g to 0.15 mg per kg body weight.

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kD. 1 2 3 4 5 6 7 8 9

53- — — — — —

FIG.1A

kD. 1 2 3 4 5 6 7 8 9

53- — — — — — — — —

FIG.1B

SUBSTITUTE SHEET

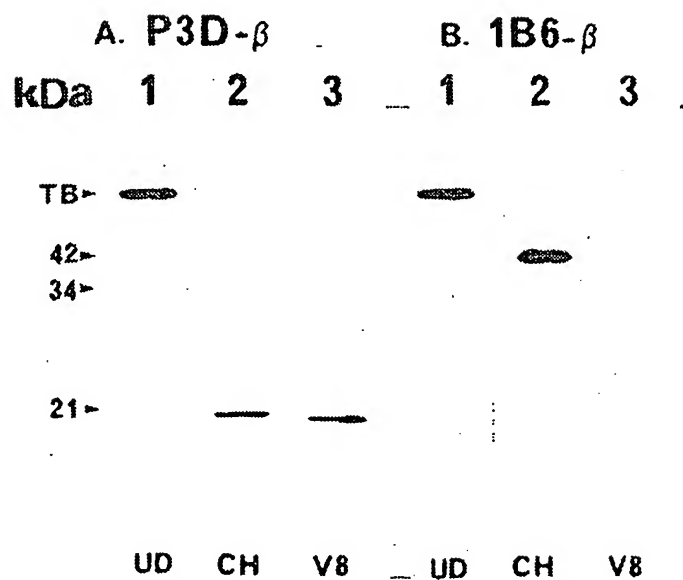
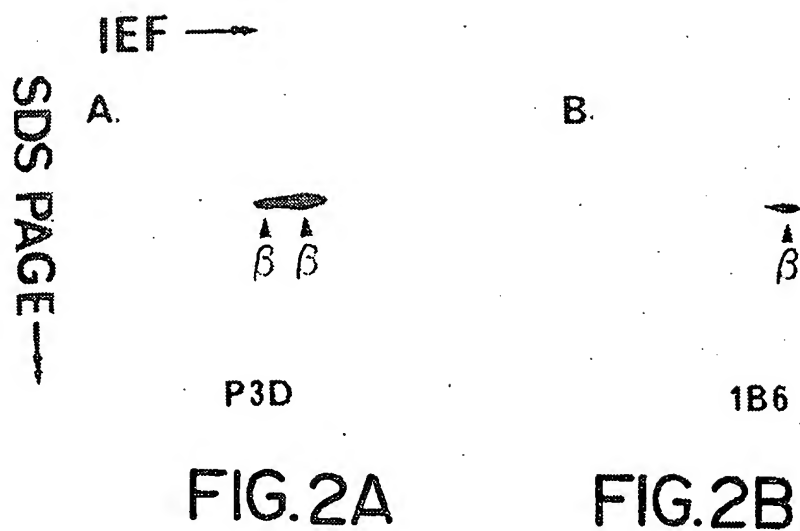


FIG.3A FIG.3B

SUBSTITUTE SHEET

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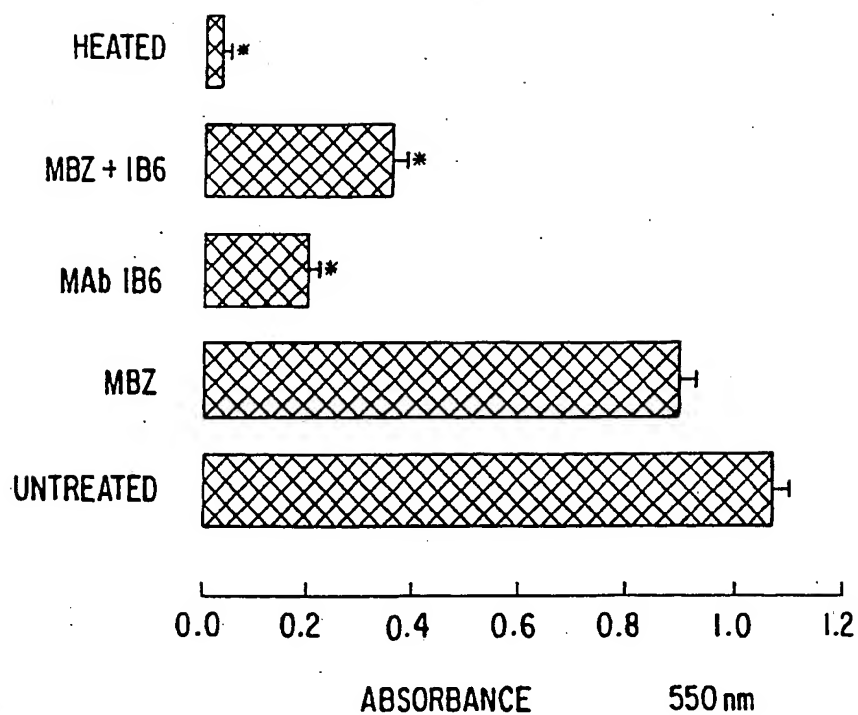


FIG. 4

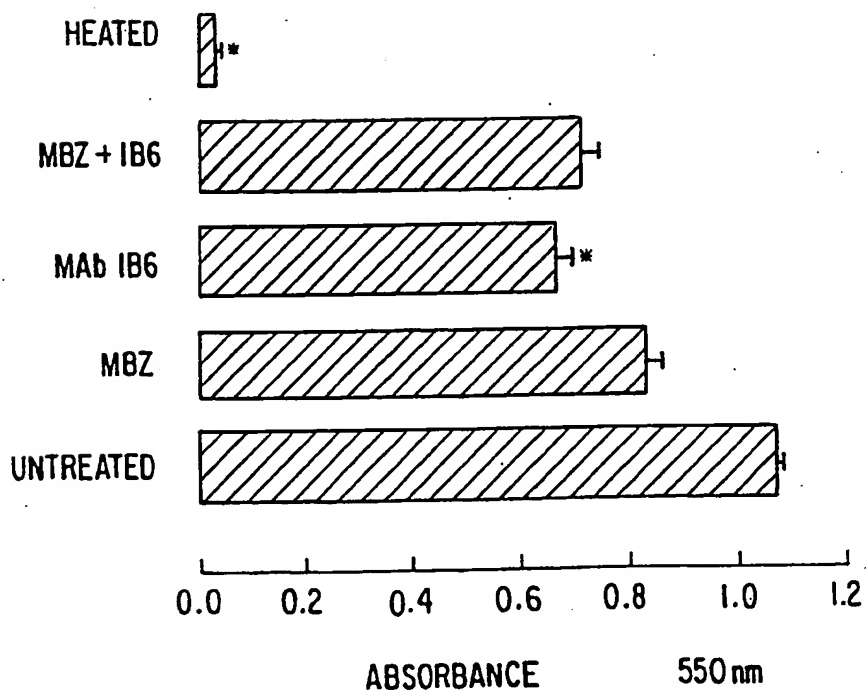


FIG. 5

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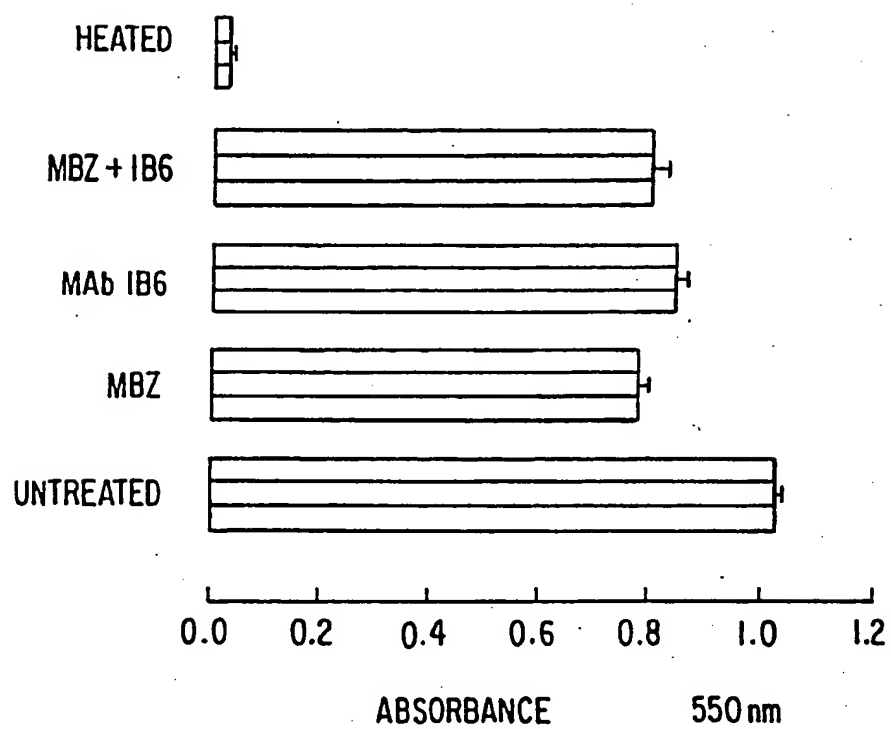


FIG.6

INTERNATIONAL SEARCH REPORT

Intern. Application No.
PCT/CA 93/00431

A. CLASSIFICATION OF SUBJECT MATTER IPC 5 C07K15/00 C12P21/08 C12N5/20 A61K39/395 G01N33/577 //A61K39/00, C07K7/08		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) IPC 5 C07K		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practical, search terms used)		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	AM. J. TROP. MED. HYG. vol. 45, no. 3SUP, September 1991 page 186 BUGHIO ET AL. 'Characterization and biological activities of monoclonal antibodies specific to nematode tubulin' & FORTITH ANNUAL MEETING OF THE AMERICAN SOCIETY OF TROPICAL MEDICINE AND HYGIENE, 1-5 /12 / 1991 BOSTON, USA	1,3,5,6
X	MOL. BIOCHEM. PARASITOL. vol. 44, no. 2, 1991 pages 153 - 164 GUENETTE ET AL. 'Characterization of a beta-tubulin gene and beta-tubulin gene products of Brugia pahangi'	1,7,8
Y	--- -/--	11,12
<div style="display: flex; justify-content: space-between;"> <input checked="" type="checkbox"/> Further documents are listed in the continuation of box C. <input checked="" type="checkbox"/> Patent family members are listed in annex. </div>		
<div style="display: flex;"> <div style="flex: 1;"> <p>* Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="flex: 1;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"&" document member of the same patent family</p> </div> </div>		
Date of the actual completion of the international search <div style="text-align: center; font-weight: bold;">18 January 1994</div>		Date of mailing of the international search report <div style="text-align: center; font-weight: bold;">03. 02. 94</div>
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016		Authorized officer <div style="text-align: center; font-weight: bold;">GAC, G</div>

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/CA 93/00431

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	GB,A,2 152 510 (KENNETH K.L.) 7 August 1985 see the whole document ---	11,12
X	WO,A,92 03549 (EURO-DIAGNOSTIC B.V.) 5 March 1992 see page 3, line 1 - line 6 see page 8, line 35 - line 39 see page 9, line 1 - line 5 ---	1,4
X	PARASITE IMMUNOLOGY vol. 11, no. 5, September 1989 pages 479 - 502 HELM ET AL. 'Localization and immunogenicity of tubulin in the filarial nematodes Brugia malayi and B. pahangi' cited in the application see page 479 see page 481 - page 482 see page 484 - page 486 see page 495 - page 498 ---	1,2
Y A	---	4 7-10
X	INTERNATIONAL JOURNAL OF PARASITOLOGY vol. 21, no. 8, 1991 pages 913 - 918 BUGHIO ET AL. 'Identification of tubulin isoforms in different tissues of Ascaris suum using antitubulin monoclonal antibodies' ---	1,2
Y	WO,A,83 01739 (BRIGHAM AND WOMEN'S HOSPITAL) 26 May 1983 see whole document, especially pages 3 and 15-19 -----	4

INTERNATIONAL SEARCH REPORT

I, international application No.

PCT/CA93/00431

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 3-10, 13-17 are directed to a method of treatment of (diagnostic method practised on) the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No.

PCT/CA 93/00431

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
GB-A-2152510	07-08-85	AU-B- 572908	19-05-88
		AU-A- 3497484	08-05-86
		DE-A- 3402492	24-10-85
		FR-A- 2573983	06-06-86
		JP-A- 60152423	10-08-85

WO-A-9203549	05-03-92	NL-A- 9001832	16-03-92
		AU-A- 8413691	17-03-92

WO-A-8301739	26-05-83	AU-A- 1044882	01-06-83
		EP-A- 0093775	16-11-83
